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Validating reference genes within a mouse model system of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity

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ABSTRACT

Background: Quantitative real-time PCR (qPCR) is the "gold-standard" technique for measuring mRNA abundances. To correctly compare samples and generate biologically valid results, qPCR data usually require comprehensive normalization to account for sample content variation between reactions. The most common normalization approaches use one or more endogenous controls (reference or house-keeping genes) to adjust the measured levels of experimental genes appropriately. Ideal reference genes are those that display minimal variation across experimental conditions, and thus can vary widely across different biological systems. In particular, toxicogenomic studies of transcriptionally-disruptive toxins, like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), require careful consideration of reference genes.

Results: We examined seven candidate reference genes in 199 mice varying in genotype and time/dose of TCDD exposure. We assessed gene-stability in four ways: (1) the variance of the raw C_q values across biological replicates, (2) the fold-change from basal mRNA levels following treatment, (3) the inter- and intra-group stability evaluated using the NormFinder algorithm, (4) the comparative ΔC_q method for each candidate gene.

Results: Univariate analyses showed *Hprt* and *Eef1a1* are the two most stable individual reference genes. It has been suggested that using multiple genes would produce a more consistent normalization factor; multivariate analysis was performed using NormFinder. In general, stability increased with the number of genes used, but specific gene-combinations synergized.

Conclusions: We have validated seven reference genes for use in analyzing mRNA abundances in mouse models of TCDD toxicity. The use of multiple reference genes increases stability, providing more consistent normalization and more reliable results. The number of reference genes used should be maximized, based on experimental capabilities (platform, sample availability, etc.). Our results show the benefit of validating reference genes using multiple methods prior to generating large biological datasets.

1. Introduction

The measurement of cellular materials, such as mRNA, has increasingly become an important tool in toxicological studies [3,17,40]. Quantitative real-time PCR (qPCR) is a fast, highly accurate, sensitive, experimentally efficient, and reproducible method of quantifying mRNA abundance [15], and has thus become the gold-standard for mRNA abundance studies. However, accurate assessment of RNA abundance, including non-coding and mRNA, requires thorough normalization of qPCR-generated data. This normalization is intended to adjust for varying amounts of starting material between reactions and different enzymatic efficiencies.

Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; qPCR, quantitative real-time PCR; C_q, quantification cycle; NEL, normalized expression level.

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It is typically performed using an endogenous "reference", which allows multiple reactions to be compared. An effective reference gene will maintain a consistent level of mRNA abundance across various tissue-types, regardless of environmental conditions. Therefore, these genes often perform a function essential for cell survival, and are sometimes called "housekeeping genes". Reference genes such as glyeraldehyde-3-phosphate dehydrogenase (*Gapdh*) and beta-actin (*Actb*) have been frequently used for qPCR data normalization [43]. However, recent studies have shown that these reference genes do not maintain stable mRNA abundance levels under many experimental conditions [11,14,39]. Rather, differences in tissues [44], experimental manipulation [36], experiment duration [48], organism (between and within species) [36] and reagents used [19] can affect the mRNA abundance levels of candidate reference genes. It is therefore essential that candidate reference genes be evaluated first under experimental conditions similar to those in the biological study.

Dioxins embody a class of environmental contaminants primarily produced through industrial processes such as electronics recycling [41], power generation, and the manufacture of herbicides and pesticides [23,37]. The most potent dioxin congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which induces highly variable toxic responses among rodent species. This makes it of particular interest to the field of toxicology. TCDD is lipid-soluble and bioaccumulates within fat stores; at current background exposure levels, it has a half-life in adult humans of approximately 7–12 years [2,25]. In animal models, exposure to even a single dose of dioxins can lead to a wasting syndrome followed by death [38]. In humans, high short-term exposure often results in liver damage and chloracne, while long-term exposure to lower levels has been linked to diabetes [20], immune deficiency [46], and a variety of cancers [4,23].

TCDD exerts its effects by acting as a ligand for the Aryl Hydrocarbon Receptor (AHR) [28]. Binding of TCDD to cytoplasmic AHR leads to formation of a ligand-receptor complex which then translocates into the nucleus and dimerizes with the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT). The ligand-AHR/ARNT complex has been shown to alter the transcriptional regulation of numerous genes [27]. Previous studies have indicated the dysregulation upwards of 3000 genes in sensitive animals following TCDD exposure [7]. Although the mechanism by which the AHR pathway alters transcription is well characterized, specific transcriptional changes which result in the biological manifestations of TCDD-mediated toxicities remain unclear. Extensive mRNA abundance studies are required in order to clarify the specific target genes responsible for the severe toxicity of TCDD.

Mouse and rat models have been used extensively to elucidate the mechanism by which AHR activation by TCDD leads to toxicity. In particular, many groups have exploiting the varying TCDD-sensitivities of different species and strains of rodents. For example, the Long-Evans rat strain (*Turku/AB*; L-E) displays a very low tolerance for TCDD (LD_{50} 10 μg/kg of TCDD) while the Han/Wistar rat (*Kuopio*; H/W) is essentially refractory to TCDD-induced lethality (LD_{50} > 9600 μg/kg) [32]. This difference in sensitivity is caused by a point mutation in the H/W *Ahr* that creates a cryptic splice-site, resulting in two isoforms of the AHR protein [33]. Although these variant proteins maintain functionality, a subset of target genes is differentially-expressed according to the specific AHR isoforms present (variant *vs.* wild-type) [13]. Thus it is hypothesized that these genes may be responsible for the manifestation of strain-specific TCDD toxicities. Similarly, different strains of mice vary in their TCDD sensitivity. Both C57BL/6 and DBA/2 mice exhibit toxic effects following TCDD exposure, but DBA/2 require a dose approximately 10 to 20 times greater than that required by C57BL/6 to induce toxic effects [10]. A point mutation in the ligand binding domain of the DBA/2 *Ahr* is the likely cause for this difference [34]. Sex also plays a role in TCDD–AHR activity, with female rats being more sensitive to TCDD-lethality than males. In mice this relationship is inverted, with females being more resistant than males [30].

It is evident that further studies investigating the TCDD-induced transcriptional changes are necessary to fully understand the various observed toxicities. However, in order for these studies to proceed, there is a pressing need for validated reference genes within these model systems. We previously identified and validated several reference genes for use in rat models of TCDD toxicity [31]. However, there are no validated reference genes for use in mouse models, despite widespread use. Since rats and mice differ dramatically in their transcriptomic responses to TCDD [6,8], it is unclear if these rat reference genes will be useful in studies using mice. Therefore, we examined here those genes previously identified as suitable reference genes for rat-TCDD studies to determine their validity in similar mouse model systems. Seven candidates (*Gapdh*, *Hprt*, *Pgk1*, *Rpl13a*, *Sdha*, *Ppia*, and *Eef1a1*) have been assessed using four separate methods for constant mRNA abundance levels in murine hepatic tissue from various mouse models treated with varying TCDD doses and collected at multiple times following exposure.

2. Methods

2.1. Animal handling

C57BL/6 mice were bred in the colonies of the National Public Health Institute, Division of Environmental Health, Kuopio, Finland. This study included female C57BL/6 wild-type mice, male C57BL/6 wild-type or transgenic mice and male DBA/2J mice. Animals were generally used at 12–15 weeks old, however the age of the transgenic mice ranged up to 23 weeks. Animals were housed with environmental conditions maintained at 21 ± 1 °C with a relative humidity of 50 ± 10% and a 12 h light cycle (12 h of light followed by 12 h of dark) in suspended, wire-mesh stainless-steel cages or in Makrolon cages with aspen chip bedding (Tapvei Oy, Kaavi, Finland). Animals were housed singly to avoid aggressive social behavior. Mice were provided with Altromin 1314 pellet feed (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and water available *ad libitum*. The study plans were approved by the Finnish National Animal Experiment Board (Eläinkoelautakunta, ELLA; permit code: ESLH-2008-07223/Ym-23).

2.2. Experimental design

Ten different experiments, comprising 199 total mice were used (Table 1). TCDD was dissolved in corn oil and administered by oral gavage (10 mL/kg). Mice treated with corn oil alone acted as controls in each experiment.

Experiments 1–5 (Fig. 1A) were time-course studies in which a single dose of TCDD was given at time zero, and mice were euthanized at different time points afterwards. Experiment E1 (n = 18) included both male and female C57BL/6 mice treated with either 0 or 500 μg/kg of TCDD and euthanized 6 h post-treatment. E2 (n = 24) consisted of male DBA/2J and transgenic C57BL/6 mice which harbored the rat wild-type allele of the *Ahr* [29]. These were treated with 0, 5 or 500 μg/kg TCDD and were euthanized after 19 h. E3 (n = 17), E4 (n = 18), and E5 (n = 17) all included both male and female C57BL/6 mice that received either 0 or 500 μg/kg TCDD. The mice from these experiments were sacrificed at 24, 72, and 144 h, respectively.

Experiments 6–10 (Fig. 1B) were dose–response studies utilizing mice treated with a single dose of 125, 250, 500, or 1000 μg/kg TCDD and then euthanized 96 h following treatment. E6

Table 1
Experimental design.

Study	Strain	Sex	Genotype	Treatment (TCDD μg/kg)	Time of tissue harvest (hours)	Number of animals
Experiment 1	C57BL/6	Male, female	WT	0, 500	6	18
Experiment 2	C57BL/6 DBA/2J	Male	rWT Ala375Val	0, 5, 500	19	24
Experiment 3	C57BL/6	Male, female	WT	0, 500	24	17
Experiment 4	C57BL/6 DBA/2 J	Male, female	WT	0, 500	72	18
Experiment 5	C57BL/6	Male, female	WT	0, 500	144	17
Experiment 6	C57BL/6	Male	WT	0, 125, 250, 500, 1000	96	20
Experiment 7	C57BL/6	Male	DEL	0, 125, 250, 500, 1000	96	21
Experiment 8	C57BL/6	Male	INS	0, 125, 250, 500, 1000	96	22
Experiment 9	C57BL/6	Male	rWT	0, 125, 250, 500, 1000	96	18
Experiment 10	C57BL/6	Female	WT	0, 125, 250, 500, 1000	96	24

Ten experiments were used in this study. Studies varied in the combination of gender, *Ahr* allele, treatment (dose(s) of TCDD) and time-points at which tissue was collected.

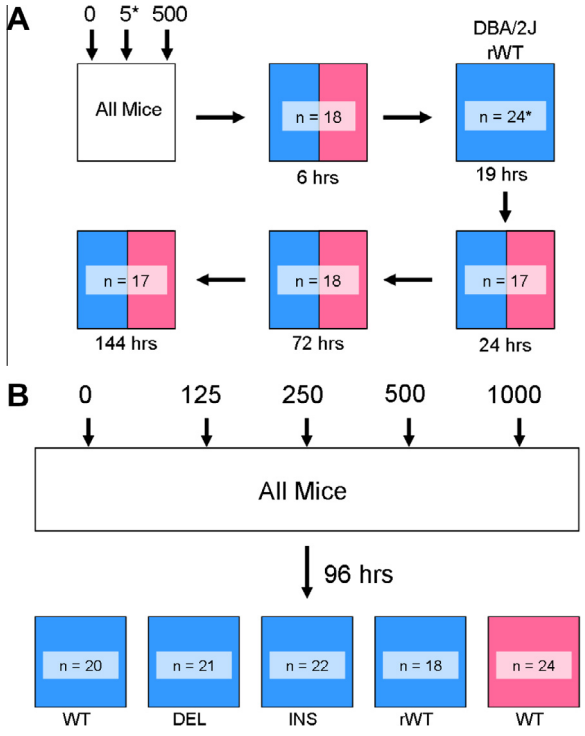

Fig. 1. Experimental design. (A) Outline of the experimental design for the time-course study. Mice were treated with either 0, 5 or 500 μg/kg TCDD in corn oil vehicle and euthanized at specific time-points following exposure. Male (blue) and female (pink) C57BL/6 mice were euthanized at 6, 24, 72 or 144 h post-exposure while male DBA/2J and ratonized-WT mice were collected at 19 h post-exposure. (B) The experimental design for the dose–response study. Male (blue) ratonized mice or female (pink) C57BL/6 mice were treated with a single dose of 0, 125, 250, 500 or 1000 μg/kg TCDD in corn oil vehicle and euthanized 96 h following exposure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

($n = 20$) consisted of male C57BL/6 mice treated as stated above. E7 ($n = 21$), E8 ($n = 22$), and E9 ($n = 18$) used male transgenic mice. E7 consisted of transgenic mice containing the H/W deletion variant (the resultant AHR protein lacks 43 amino acids at position 766) [26]. E8 included mice containing the H/W insertion variant of the *Ahr* gene (resulting in an AHR isoform with an insertion of 7 novel amino acids at position 808 and a lack of the remaining 45 amino acids at the C-terminus) [26]. E9 consisted of mice harboring the rat wild-type *Ahr*. Finally, E10 ($n = 24$) consisted of female wild-type C57BL/6 mice.

All animal handling and reporting comply with ARRIVE guidelines [16].

2.3. RNA isolation

In all cases, mouse livers were excised and snap-frozen in liquid nitrogen following euthanasia by carbon dioxide exposure. Liver tissue was shipped on dry ice to the analytical laboratory and stored at −80 °C or colder. Prior to RNA isolation, tissue samples were ground to a fine powder in liquid nitrogen using a mortar and pestle followed by addition of lysis buffer and rapid homogenization using a Brinkmann Polytron (Polytron PT1600E with a PT-DA 1607 generator). An RNeasy Mini Kit (Qiagen, Mississauga, Canada) was used to extract RNA from the homogenized samples according to the manufacturer's instructions. Quantification of total RNA was performed using a NanoDrop UV spectrophotometer (Thermo Scientific, Mississauga, ON). RNA integrity was determined by electrophoresis using RNA 6000 Nano kits on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All RNA samples had an RNA integrity number above 8.5 and were used in downstream analyses. cDNA was generated using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA) following the manufacturer's recommended protocol and the product was diluted to produce a final concentration of 5 ng/μL cDNA.

2.4. Quantitative PCR

qPCR was carried out in 384-well plates on the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems) using the manufacturer's default settings for absolute quantification of standard C_q. Each 10 μL reaction contained 5 ng cDNA, 1× gene-specific PrimeTime assay (Supplementary Table 1; Integrated DNA Technologies, Corralville, IA), and TaqMan Gene Expression Master Mix (Applied Biosystems). Pre-designed PrimeTime assays were used (Integrated DNA Technologies; January 2012). Reaction efficiencies for each primer/probe set were determined to be between 82% and 102%. Every reaction was performed in at least duplicate. Samples were assessed for the presence of genomic DNA amplification by including a reaction that did not contain reverse transcriptase, and these were run in parallel with each cDNA sample assayed. Similarly, no-template controls were run with each primer/probe set used. Briefly, the PCR consisted of an initial incubation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Baseline and threshold values were automatically detected using the Sequence Detection System Software v2.3 (Applied Biosystems) with default settings. The above qPCR processes and subsequent reporting all comply with MIQE guidelines [9].

2.5. Western analysis

Protein levels for candidate genes were determined by quantitative western blotting of mouse liver protein. Briefly, total protein

was isolated from each sample using Tissue Extraction Reagent (Invitrogen, Burlington, ON) supplemented with cOmplete protease inhibitor cocktail (Roche, Laval, QC). Protein extract was quantified by Bradford assay and diluted to a concentration of 10 μg/μL. A total of 10 μg protein extract was loaded into each well of a Novex 4–12% Bis–Tris gel system and electrophoresed for 40 min at 200 V with MES running buffer (Invitrogen). Proteins were transferred to PVDF membranes using the iBlot system program P0 for 7 min (Invitrogen). Primary antibodies were purchased from Santa Cruz and were used at a 1:500 dilution in Li-Cor blocking buffer, with overnight incubation at 4 °C. Blots were washed three times with PBS supplemented with 0.1% tween 20 at room temperature for 5 min. The Li-Cor IRDye-labeled secondary antibodies were purchased from Mandel Scientific and used at a dilution of 1:10,000 in Li-Cor blocking buffer and incubated at room temperature for 1 h. After washing the blots as described, they were analyzed with the Odyssey (Li-Cor Biosciences, Lincoln, NE, USA) quantitative western blot near-infrared system using default settings.

2.6. Statistical analyses and visualization

Collected C_q values were loaded in the R statistical environment (v2.15.1) for all analyses. The mean C_q with standard deviation for each candidate gene was determined for each experimental condition. These values were then used to determine the mean and standard deviation of the C_q values across all conditions, and standard deviations were used to identify the candidate with the least variation, as suggested by ABI [18]. A similar method has been used to evaluate several of our candidate reference genes within human CD4+ T cells and monocytes [21]. The $\log_2$ |fold-change| of each reference gene was calculated as the difference in C_q values between treated and control animals. In addition, two popular algorithms, NormFinder [1] and the comparative ΔC_q method [42], were used to determine the most stable reference gene. Candidate genes were analyzed both individually and in all possible combinations. All possible combinations of the seven candidate genes were arranged using the gregmisc (v2.1.2) package for R and all visualizations were produced using the lattice (v0.20-10) and latticeExtra (v0.6-24) R packages.

The mRNA abundances were assessed across both the time-course and the dose–response experiments. Each treatment group (biological replicate) was compared to a control group of the same gender, strain, genotype and time-point, which was not exposed to TCDD. Unpaired, two-tailed Student's *t*-tests were used to evaluate differential mRNA abundance levels between control and treatment groups using the C_q values for each individual candidate reference gene. The magnitude (M) of the differences was evaluated using the $\log_2$|fold-change| and defined as follows: $[M = \log_2(2^{\Delta C_q}),$ where $\Delta C_q = C_{q(\mathrm{TCDD})} - C_{q(\mathrm{Cornoil})}]$. Both individual genes and each combination of genes were assessed. Results were visualized as $M \pm$ standard-deviation across all experimental conditions.

The NormFinder algorithm [1] estimates the overall variation of mRNA abundance levels for a candidate reference gene by analyzing its variance both within an experimental group and across experimental conditions. The geometric mean of the C_q values for technical replicates was calculated and transformed as follows: $[C_q = 2^{-geometric\ mean\ (C_q)}]$. Samples were identified as belonging to either control or treatment groups and were evaluated using the NormFinder algorithm in the R statistical environment (v2.15.1). Interpretation of NormFinder output is as follows: a lower value indicates more consistent mRNA abundance levels across experimental groups signifying a potentially good reference gene. Profiles utilizing multiple candidate genes were evaluated using the geometric mean of the C_q values for the pertinent genes.

The comparative ΔC_q method was used to compare C_q values between each pair of candidate genes within each sample. The candidate with the least variation (the smallest mean standard deviation across comparisons) was considered to be the most stably expressed [42].

3. Results

Experiments were divided into two categories: time-course (Fig. 1A) and dose–response (Fig. 1B) studies. qPCR findings were validated by considering mRNA abundance levels determined by microarray data from a subset of these animals; similar results were obtained (data not shown).

3.1. Univariate comparison of genes

We first evaluated the variation of raw C_q values for each candidate gene across all experimental conditions (Table 2, Supplementary Fig. 1). *Hprt* had the smallest standard deviation across all experiments while *Sdha* had the largest (average $\sigma = 0.50$ and 1.40, respectively). As this increased variation may result from TCDD-mediated alteration of mRNA levels, we next assessed the differential expression between treated and untreated animals.

The extent of TCDD-treatment on the mRNA levels of the candidate genes can be assessed from the fold-difference between basal and TCDD-altered mRNA levels. This was calculated for each gene at each treatment condition (Supplementary Table 2). The fold-changes for two representative candidate genes, *Eef1a1* and *Gapdh*, are shown for all time-course (Fig. 2A) and dose–response (Fig. 2B) experiments. Similar plots for the fold-change in mRNA levels of the five remaining candidate genes are shown in Supplementary Fig. 2. Given that an ideal reference gene will maintain a consistent level of mRNA abundance, regardless of experimental conditions, the more suitable reference gene will be one with minimal fold-change variation within and between experimental conditions. Unpaired Student's *t*-tests were performed to assess the statistical significance of the fold-changes for each gene at each experimental condition (Supplementary Table 2). This analysis indicated that *Eef1a1* displayed the least variation between treated and untreated animals (9/33 experimental conditions with $p < 0.05$). In contrast, 26/33 experimentally unique groups had significantly differential expression of *Rpl13a* between treated and untreated animals (Table 2). Pearson's correlations were then used to assess the similarity of variation across experimental conditions between all genes (Fig. 3). *Hprt*, *Ppia*, and *Rpl13a* are well correlated, as are *Gapdh* and *Sdha*, indicating similar direction of influence by TCDD-treatment.

The above approaches were applied independently to each combination of candidate gene and experimental condition. The distributions of these assessments were then compared across genes to determine overall trends. To address this comparison of conditions in a more global and quantitative fashion, we evaluated the gene stability using the NormFinder algorithm [1]. Unlike the prior methods, this approach requires a relatively large number of samples and recommends the assessment of 5–10 candidate genes simultaneously in order to produce valid results. NormFinder ranks each gene based on its intra- and inter-group variation in mRNA abundance levels, as well as the systematic differences between groups. This value represents the amount of variation: candidates with a higher value display greater variation (instability) across experiments; however, it is important to note that the magnitude of these values is less significant than the ranking of the candidate genes. In order to validate the reproducibility of the candidate rankings, experiments were randomly divided into two datasets: the training set included experiments 1, 4, 6, 8, and 9

Table 2
Summary of analysis methods.

	C_q variation (ABI)		Unpaired Student's *t*-test	NormFinder		Comparative ΔC_q
	Variation within groups biological replicates		Comparison between groups biological replicates	Training 96 Animals inter-group and intra-group variance	Validation 103 Animals inter-group and intra-group variance	Comparison between genes technical replicates
Eef1a1	3.32	0.88	9/32	0.098	0.181	1.35
Gapdh	4.83	1.25	18/32	0.121	0.128	1.50
Hprt	2.00	0.50	17/32	0.089	0.137	1.29
Pgk1	5.28	0.99	11/32	0.120	0.171	1.35
Ppia	3.23	0.84	19/32	0.115	0.154	1.54
Rpl13a	4.93	1.18	26/32	0.148	0.166	1.55
Sdha	6.57	1.40	16/32	0.183	0.203	1.76

Four separate analyses were used to assess the stability of each individual candidate gene: (1) C_q variation (range, StDEV) was calculated across all experimental groups. (2) Student's *t*-tests were performed between treated and untreated animals for each experimental condition; groups with a significant *p*-value (<0.05) were determined to be altered by TCDD. (3) NormFinder assessed the consistency of each candidate using both the inter- and intra-group variation. (4) The comparative ΔC_q method assessed the affect of normalization of all genes using each candidate; the average standard deviation for each pair-wise comparison is reported.

while the validation set included the remainder (experiments 2, 3, 5, 7, and 10) (n = 96 and 103, respectively). NormFinder was then run independently upon each dataset and results were compared between them (Fig. 4, Supplementary Table 3). The two sets of experiments generated only partially-similar results: *Hprt* and *Eef1a1* were the most stable genes in the training dataset, while *Hprt* and *Gapdh* were identified in the validation dataset. *Sdha* was indicated as the least stable candidate in both datasets (Table 2).

To this point all candidate genes were assessed individually across varying experimental conditions. Alternatively, the variability of a candidate gene can be assessed through its capacity to generate consistent normalized expression levels of other candidate genes. The normalized expression level (NEL) of a given mRNA is typically calculated using the difference in C_q from the reference. Any variation between two candidate genes across experimental conditions indicates variation in one or both of those genes, and can be used to determine overall stability [44]. To this end, we performed analysis of each candidate gene using the comparative ΔC_q method [42] (adapted from geNorm [44]) to determine the overall variance when compared to all other candidate genes, producing similar results as the above methods. The difference between C_q values (ΔC_q) for each pair of candidate genes was calculated for each individual sample. The mean and standard deviation for these values was calculated across all samples. Finally, the mean of the standard deviations was used to determine the variation for each candidate gene. By this method, *Hprt*, *Eef1a1*, and *Pgk1* were the least variable across experimental conditions (Table 2, Supplementary Table 4).

3.2. Multivariate analysis

Lastly, we sought to consider how these genes could work together as a reference set, which may be more effective because not all genes in a set are likely to be altered by the same experimental condition [44]. To test this, the stability of all possible combinations of the candidate genes in this study was assessed using the NormFinder algorithm. NormFinder was used to rank all possible combinations of the seven candidate genes using the geometric mean of their C_q values for each treatment group. As above, experiments were divided into training and validation sets and NormFinder was run independently on each (Fig. 5). Ideal combinations of reference genes will have smaller instability values (thus having increased stability) and be reproducible in the validation set.

Generally, it was found that stability increased with a larger number of genes input into NormFinder (Spearman's rho: −0.68, $p < 0.01$) (Fig. 5A). In fact, the greatest stability was achieved by using all seven candidate genes. Although using more genes generally improved stability, certain combinations of genes performed

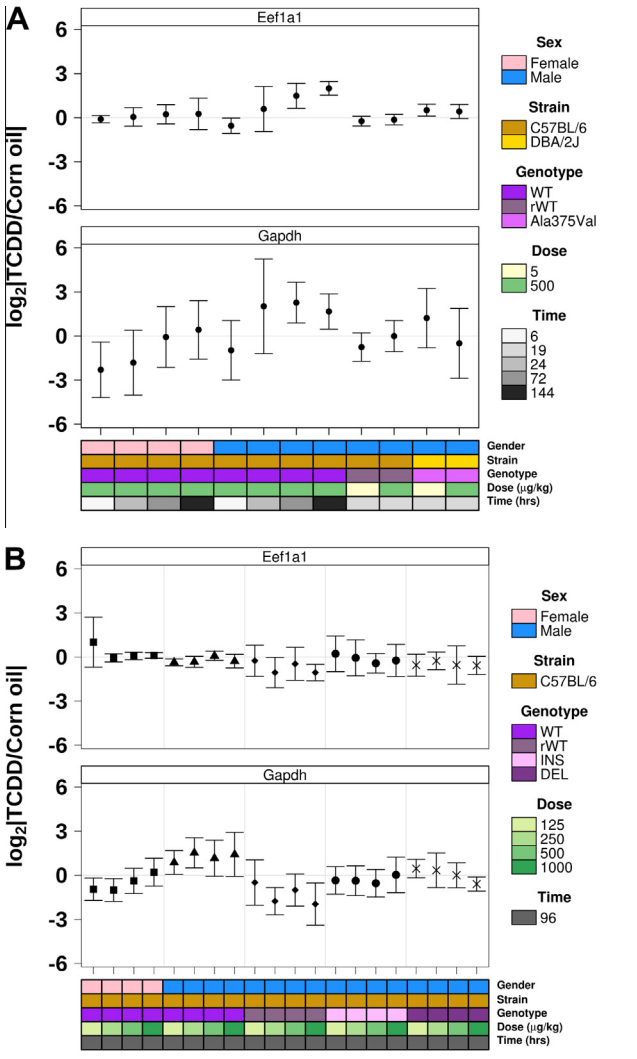

Fig. 2. Time-course and dose–response of *Eef1a1* and *Gapdh*. Fold changes were calculated and compared across all conditions present in the (A) time-course and (B) dose–response studies. Candidate reference genes *Eef1a1* and *Gapdh* are shown. Points represent the fold change (in $\log_2$ space) of the TCDD-treated animals compared with the control animals of the same biological conditions. Error bars indicate the standard deviation within each group. Different point types (B) indicate separate dose–response studies.

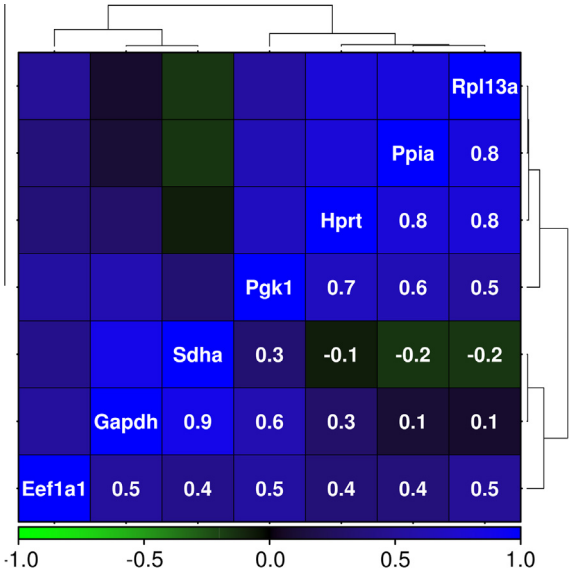

Fig. 3. Correlation of candidate genes. Fold changes were calculated for each experimental condition and Pearson's correlations used to compare each pair of candidate genes. Correlations were visualized using a heatmap and organized using divisive clustering. Blue indicates perfect correlation; green represents inverse correlations while black indicates little or no correlation. *R* values are shown in white for each pair-wise comparison. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

better than others. For example, not all possible groups of 6 genes outperformed all combinations of 2–5 genes and many combinations of genes performed differently in the training and validation sets (Fig. 5B). Similarly, certain groups of candidates ranked differently between the two datasets (Fig. 5C), with the inclusion of certain candidates contributing more than others to increased stability (Supplementary Fig. 3).

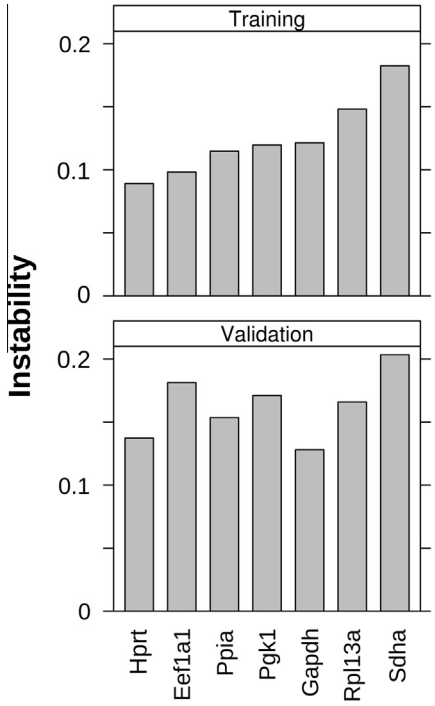

Fig. 4. Univariate analysis of gene stability by NormFinder. Experiments were separated into training and validation sets, ensuring similar treatment conditions appeared in both sets. Each gene was analyzed using the NormFinder algorithm (see *Section* 2.6) to determine stability across all treatment groups. A lower value indicates less variance across all experimental conditions.

To verify the multivariate results, two of the top combinations of genes, as determined by NormFinder for both the training and validation sets, were visualized across all experimental conditions for both time-course and dose–response studies (Fig. 6A and B, respectively). The most stable combination included all seven candidate genes, followed closely by this combination without *Hprt*. This is despite our findings that *Hprt* is the most stable individual gene in our study, indicating that the number of genes used for normalization of qPCR data is more relevant than the identity of those genes.

4. Discussion

Quantitative real-time PCR is the "gold-standard" for determining mRNA abundance due to its high accuracy and sensitivity. However, accurate interpretation of results requires data normalization typically using stable endogenous controls. Suitable reference genes are those proven to maintain stable transcript levels across all experimental treatments and conditions. Failure to select and validate appropriate reference genes may cause inaccurate interpretation of results. In fact, it is seldom appreciated that most studies (including ours) do not describe reference genes *per se*, but rather specific target regions of that gene that are stable under specified conditions: different splice-variants or isoforms may respond differently to experimental conditions [12]. Similarly, validity of reference genes may not be consistent between RNA and protein analyses: these two species are only weakly correlated [22]. For example, in the samples used in this study, matched mRNA and protein levels for Eef1a1 are uncorrelated across experimental conditions ($\rho = -0.16$, $p > 0.01$; Supplementary Fig. 4, Supplementary Table 5). Therefore, it is extremely important for candidate reference genes to first be validated across all experimental conditions. The applicability of results discussed here are dependent on the specific gene regions and qPCR assays reported in Supplementary Table 1, although of course may be extensible to other untested regions.

The toxic effects of TCDD have been shown to be mediated through its interaction with the AHR which results in alteration of mRNA abundance levels for numerous genes [27]. The exact transcriptional changes responsible for the resultant toxic effects of TCDD are still unknown; therefore, further study is required. Due to this large effect of TCDD on transcription, it is imperative that potential reference genes are first evaluated in the relevant animal models. Reference genes for rat models of TCDD toxicity have been previously identified [31], however, there has yet to be a study validating candidate reference genes in mouse models treated with TCDD. Similarly, as there is a large degree of inter-strain heterogeneity within rat models exposed to TCDD [5,47], mRNA abundance profiles may vary between different mouse models. While phenotypic and sequence homology suggest a general tendency for reference genes to remain suitable across strains or species, this is not guaranteed and, in fact, our study has clearly demonstrated this.

This study was designed to determine whether reference genes previously validated in rat models treated with TCDD would be suitable for the normalization of RNA abundance data in mouse models of TCDD toxicity. Abundance of mRNA for the seven candidate reference genes was determined for numerous mouse models, including mice of different genders, strains and *Ahr*-genotypes, which were treated with varying doses of TCDD for various time points. mRNA abundance data were generated using qPCR and analyzed to identify differences across experimental conditions in all mouse models used. Four methods were employed: (1) the

variation of the raw C_q values [18], (2) the abundance fold-change between treated and untreated animals, (3) the NormFinder algorithm for gene stability [1] and (4) the differential abundance between each pair of candidate genes (comparative ΔC_q) [42,44]. While each method used different approaches, they generated similar rankings of candidate genes (summarized in Table 2).

Since some variation is expected for any given gene across biologically identical samples, it may be more appropriate to compare levels of mRNA abundance between treated and untreated animals. Ideally, suitable reference genes will display minimal TCDD-mediated abundance differences. We performed unpaired Student's *t*-tests and evaluated the fold-difference between treated and untreated animals of identical origins (strain, gender, *Ahr*-genotype) and determined that *Eef1a1* showed the least variation across all treatment regimens (Fig. 2) while *Rpl13a* displayed the most variance.

An alternative to the analysis of the fold-difference between basal and treated mRNA levels, the comparative ΔC_q method examined the fold-difference between each pair of candidate genes in order to determine the extent of treatment-induced alteration between genes. If experimental conditions induced alterations in one gene while leaving another unaffected, the variation would be greater than if both genes were similarly altered. This method established that *Hprt* displayed minimal variation, indicating that it is the most stable candidate while *Sdha* was again the least.

The NormFinder algorithm considers variation within and across experimental groups to determine the overall stability of a candidate gene. NormFinder analysis indicated that *Hprt* was indeed among the most stable of our candidates (displaying greater stability compared to other candidates in both the training experiments and the validation set) and that *Sdha* was the least stable, regardless of experimental conditions (Fig. 4).

The use of multiple endogenous genes has been shown to generate more accurate results, with general opinion that using the geometric mean of a minimum of three stable, non-co-regulated genes would be most suitable [42,44]. Therefore, we evaluated all possible permutations of our candidate genes using the NormFinder algorithm. This analysis confirmed that, in general, combining multiple genes produces a more stable value for normalization. In fact, the use of a larger number of genes is far more critical than the selection of any specific individual gene, likely up to a maximal number not determined here. For example, the most stable

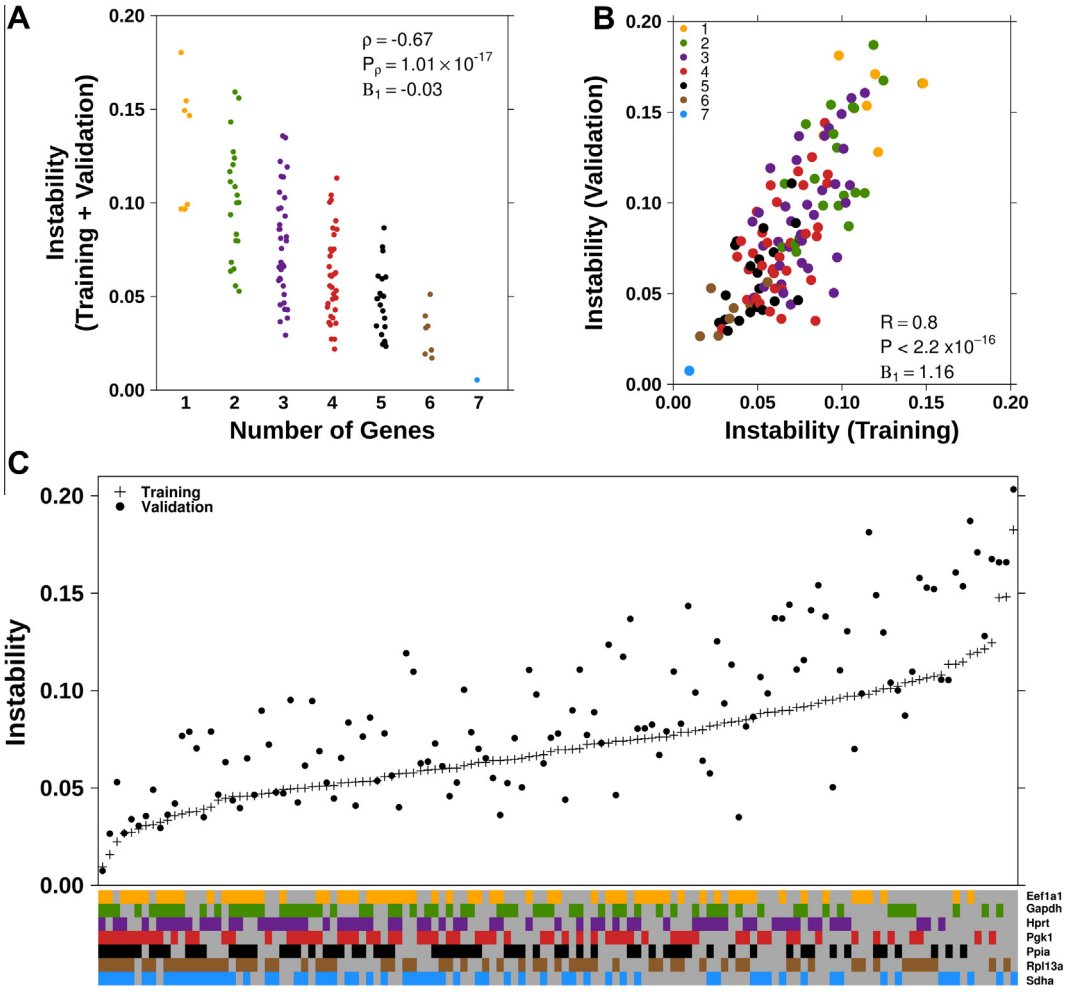

Fig. 5. Multivariate analysis of gene stability by NormFinder. Experiments were separated into training and validation sets, ensuring similar treatment conditions appeared in both sets. Each gene, and all possible combinations of genes, was analyzed using the NormFinder algorithm (see *Section* 2.6) to determine stability across all treatment groups. A lower value indicates less variance across all experimental conditions. (A) Combinations were organized according to the number of included candidates in order to determine the optimal number of genes. (B) Results for each combination of candidates were compared between the training and validation sets to visualize concordance. Points represent the stability values generated for each combination and are grouped according to the number of genes used. (C) Results for each combination of gene(s) were plotted for both the training and validation experiments. Combinations are organized according to performance in the training set: a lower value indicates less variance across experimental conditions.

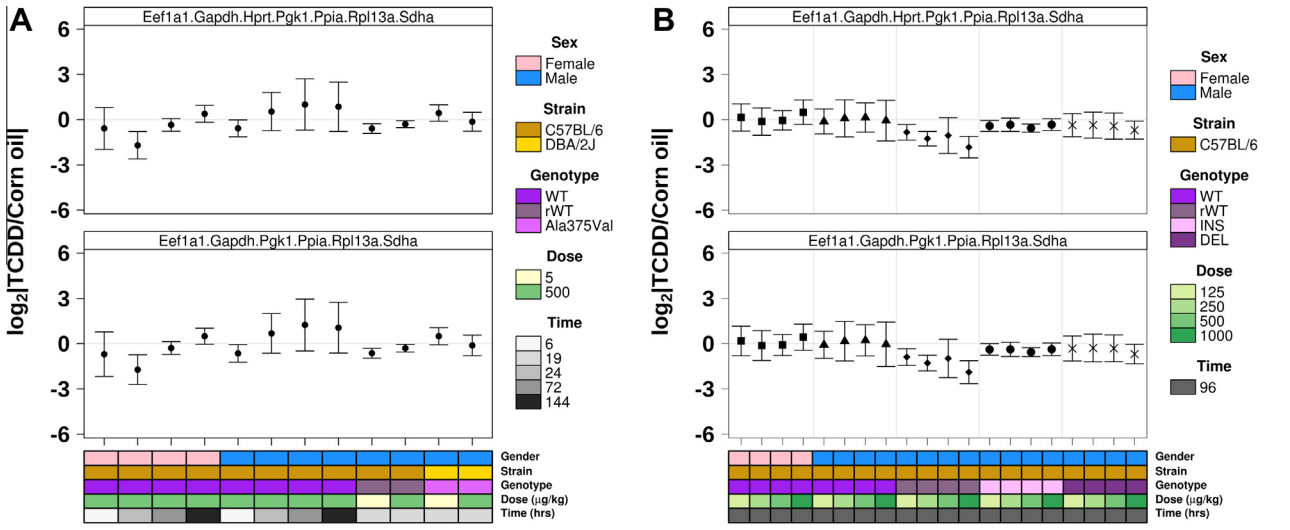

Fig. 6. Multivariate analysis of time-course and dose–response experiments. Fold changes were calculated using the geometric mean of multiple genes and compared across all conditions present in the (A) time-course and (B) dose–response studies. Points represent the fold change (in $\log_2$ space) across a single biological condition with error bars to indicate the standard deviation within the group. The geometric mean of either all seven genes assessed (top) or the best group of six genes (bottom) are shown.

combination of six genes actually excludes the single most univariately stable gene (*Hprt*). Additionally, the use of seven genes is more stable than any combination of six genes, further highlighting this phenomenon (Fig. 5B). This suggests that the number of reference genes implemented is the most significant factor; the inclusion of more genes is better and outweighs the specific genes used. However, if using a combination of six or seven reference genes is not experimentally feasible, it is possible to implement a carefully selected combination of fewer genes while still upholding stability: the most stable pair of genes, *Gapdh* and *Ppia*, demonstrated less variation than several groups consisting of a greater number of genes.

5. Conclusions

Although several new methods of interrogating mRNA abundances are being generated, these still generally require the use of control genes for data analysis [35,45]. The results of this study indicate that not all genes which remain stable in rats treated with TCDD are optimal reference genes in a murine model. In particular, while both *Rpl13a* and *Sdha* were shown to be unchanged following TCDD-exposure in rat liver [31], they appear to be the least stably expressed genes within the hepatic tissue of mouse systems. Similarly, although *Gapdh* is a commonly used reference gene and was stably expressed in rats treated with TCDD, it was only moderately stable within the mouse models. Additionally, *Gapdh* mRNA abundance increases 4-fold in response to TCDD in human keratinocytes [24]. The individual reference genes which proved to be the most stable were *Hprt* and *Eef1a1*; however, using certain combinations of two or more genes was more stable than any individual gene and the use of all seven candidate genes achieved the highest stability overall. Through this study, we found that the most significant factor surrounding reference gene selection is the number of reference genes implemented. This work provides a template for identifying reference genes for studying specific toxicological phenomena.

Authors' contributions

Animal handling: RP.
Sample preparation: SDP, JDW, RP.
Data generation: SDP.
Performed statistical and bioinformatics analyses: SDP, NSF.
Generated statistical and bioinformatics tools: SDP, NSF, LCC, DYFM.
Wrote the first draft of the manuscript: SDP, PCB.
Initiated the project: RP, PCB.
Supervised research: AP, RP, PCB.
Approved the manuscript: all authors.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2013.06.008.

References

[1] C.L. Andersen, J.L. Jensen, T.F. Orntoft, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Res. 64 (2004) 5245–5250.

[2] ATSDR, Toxicological profile for chlorinated dibenzo-*p*-dioxins, in: U.S.D.o.H.a.H. Services (Ed.), Agency for Toxic Substances and Disease Registry, Atlanta, GA, 1998.
[3] H. Badghisi, D.C. Liebler, Sequence mapping of epoxide adducts in human hemoglobin with LC–tandem MS and the SALSA algorithm, Chem. Res. Toxicol. 15 (2002) 799–805.
[4] P.A. Bertazzi, C. Zocchetti, S. Guercilena, D. Consonni, A. Tironi, M.T. Landi, A.C. Pesatori, Dioxin exposure and cancer risk: a 15-year mortality study after the "Seveso accident", Epidemiology 8 (1997) 646–652.
[5] P.C. Boutros, I.D. Moffat, A.B. Okey, R. Pohjanvirta, MRNA levels in control rat liver display strain-specific, hereditary, and AHR-dependent components, PLoS ONE 6 (2011) e18337.
[6] P.C. Boutros, R. Yan, I.D. Moffat, R. Pohjanvirta, A.B. Okey, Transcriptomic responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in liver: comparison of rat and mouse, BMC Genomics 9 (2008) 419.
[7] P.C. Boutros, C.Q. Yao, J.D. Watson, A.H. Wu, I.D. Moffat, S.D. Prokopec, A.B. Smith, A.B. Okey, R. Pohjanvirta, Hepatic transcriptomic responses to TCDD in dioxin-sensitive and dioxin-resistant rats during the onset of toxicity, Toxicol. Appl. Pharmacol. 251 (2011) 119–129.
[8] D.R. Boverhof, L.D. Burgoon, C. Tashiro, B. Sharratt, B. Chittim, J.R. Harkema, D.L. Mendrick, T.R. Zacharewski, Comparative toxicogenomic analysis of the hepatotoxic effects of TCDD in Sprague Dawley rats and C57BL/6 mice, Toxicol. Sci. 94 (2006) 398–416.
[9] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611–622.
[10] D.E. Chapman, C.M. Schiller, Dose-related effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J and DBA/2J mice, Toxicol. Appl. Pharmacol. 78 (1985) 147–157.
[11] E. Deindl, K. Boengler, N. van Royen, W. Schaper, Differential expression of GAPDH and beta3-actin in growing collateral arteries, Mol. Cell. Biochem. 236 (2002) 139–146.
[12] M.H. Falahatpisheh, K.S. Ramos, Ligand-activated *Ahr* signaling leads to disruption of nephrogenesis and altered Wilms' tumor suppressor mRNA splicing, Oncogene 22 (2003) 2160–2171.
[13] M.A. Franc, I.D. Moffat, P.C. Boutros, J.T. Tuomisto, J. Tuomisto, R. Pohjanvirta, A.B. Okey, Patterns of dioxin-altered mRNA expression in livers of dioxin-sensitive versus dioxin-resistant rats, Arch. Toxicol. 82 (2008) 809–830.
[14] E.M. Glare, M. Divjak, M.J. Bailey, E.H. Walters, Beta-actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels, Thorax 57 (2002) 765–770.
[15] C.A. Heid, J. Stevens, K.J. Livak, P.M. Williams, Real time quantitative PCR, Genome Res. 6 (1996) 986–994.
[16] C. Kilkenny, W.J. Browne, I.C. Cuthill, M. Emerson, D.G. Altman, Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research, PLoS Biol. 8 (2010) e1000412.
[17] J. Lamb, E.D. Crawford, D. Peck, J.W. Modell, I.C. Blat, M.J. Wrobel, J. Lerner, J.P. Brunet, A. Subramanian, K.N. Ross, M. Reich, H. Hieronymus, G. Wei, S.A. Armstrong, S.J. Haggarty, P.A. Clemons, R. Wei, S.A. Carr, E.S. Lander, T.R. Golub, The connectivity map: using gene-expression signatures to connect small molecules, genes, and disease, Science 313 (2006) 1929–1935.
[18] LifeTechnologies, Using TaqMan Endogenous Control Assays to Select an Endogenous Control for Experimental Studies, Life Technologies Corporation, 2012.
[19] J. Linden, J. Ranta, R. Pohjanvirta, Bayesian modeling of reproducibility and robustness of RNA reverse transcription and quantitative real-time polymerase chain reaction, Anal. Biochem. 428 (2012) 81–91.
[20] M.P. Longnecker, J.E. Michalek, Serum dioxin level in relation to diabetes mellitus among Air Force veterans with background levels of exposure, Epidemiology 11 (2000) 44–48.
[21] V.P. Mane, M.A. Heuer, P. Hillyer, M.B. Navarro, R.L. Rabin, Systematic method for determining an ideal housekeeping gene for real-time PCR analysis, J. Biomol. Tech. 19 (2008) 342–347.
[22] S. Marguerat, A. Schmidt, S. Codlin, W. Chen, R. Aebersold, J. Bahler, Quantitative analysis of fission yeast transcriptomes and proteomes in proliferating and quiescent cells, Cell 151 (2012) 671–683.
[23] N. Marinkovic, D. Pasalic, G. Ferencak, B. Grskovic, A. Stavljenic Rukavina, Dioxins and human toxicity, Arh. Hig. Rada Toksikol. 61 (2010) 445–453.
[24] S.E. McNulty, W.A. Toscano Jr., Transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Biochem. Biophys. Res. Commun. 212 (1995) 165–171.
[25] J.E. Michalek, R.C. Tripathi, S.P. Caudill, J.L. Pirkle, Investigation of TCDD half-life heterogeneity in veterans of Operation Ranch Hand, J. Toxicol. Environ. Health 35 (1992) 29–38.
[26] I.D. Moffat, S. Roblin, P.A. Harper, A.B. Okey, R. Pohjanvirta, Aryl hydrocarbon receptor splice variants in the dioxin-resistant rat: tissue expression and transactivational activity, Mol. Pharmacol. 72 (2007) 956–966.
[27] A.B. Okey, An aryl hydrocarbon receptor odyssey to the shores of toxicology: the Deichmann Lecture, International Congress of Toxicology-XI, Toxicol. Sci. 98 (2007) 5–38.
[28] A.B. Okey, D.S. Riddick, P.A. Harper, The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds, Toxicol. Lett. 70 (1994) 1–22.
[29] R. Pohjanvirta, Transgenic mouse lines expressing rat AH receptor variants–a new animal model for research on AH receptor function and dioxin toxicity mechanisms, Toxicol. Appl. Pharmacol. 236 (2009) 166–182.
[30] R. Pohjanvirta, H. Miettinen, S. Sankari, N. Hegde, J. Linden, Unexpected gender difference in sensitivity to the acute toxicity of dioxin in mice, Toxicol. Appl. Pharmacol. 262 (2012) 167–176.
[31] R. Pohjanvirta, M. Niittynen, J. Linden, P.C. Boutros, I.D. Moffat, A.B. Okey, Evaluation of various housekeeping genes for their applicability for normalization of mRNA expression in dioxin-treated rats, Chem. Biol. Interact. 160 (2006) 134–149.
[32] R. Pohjanvirta, M. Unkila, J. Tuomisto, Comparative acute lethality of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin in the most TCDD-susceptible and the most TCDD-resistant rat strain, Pharmacol. Toxicol. 73 (1993) 52–56.
[33] R. Pohjanvirta, J.M. Wong, W. Li, P.A. Harper, J. Tuomisto, A.B. Okey, Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-resistant rat strain, Mol. Pharmacol. 54 (1998) 86–93.
[34] A. Poland, D. Palen, E. Glover, Analysis of the four alleles of the murine aryl hydrocarbon receptor, Mol. Pharmacol. 46 (1994) 915–921.
[35] S.D. Prokopec, J.D. Watson, D.M. Waggott, A.B. Smith, A.H. Wu, A.B. Okey, R. Pohjanvirta, P.C. Boutros, Systematic evaluation of medium-throughput mRNA abundance platforms, RNA 19 (2013) 51–62.
[36] S. Ren, F. Zhang, C. Li, C. Jia, S. Li, H. Xi, H. Zhang, L. Yang, Y. Wang, Selection of housekeeping genes for use in quantitative reverse transcription PCR assays on the murine cornea, Mol. Vis. 16 (2010) 1076–1086.
[37] A. Schecter, L. Birnbaum, J.J. Ryan, J.D. Constable, Dioxins: an overview, Environ. Res. 101 (2006) 419–428.
[38] M.D. Seefeld, S.W. Corbett, R.E. Keesey, R.E. Peterson, Characterization of the wasting syndrome in rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Toxicol. Appl. Pharmacol. 73 (1984) 311–322.
[39] S. Selvey, E.W. Thompson, K. Matthaei, R.A. Lea, M.G. Irving, L.R. Griffiths, Beta-actin – an unsuitable internal control for RT-PCR, Mol. Cell. Probes 15 (2001) 307–311.
[40] Y.M. Shah, K. Morimura, Q. Yang, T. Tanabe, M. Takagi, F.J. Gonzalez, Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation, Mol. Cell. Biol. 27 (2007) 4238–4247.
[41] C. Shen, Y. Chen, S. Huang, Z. Wang, C. Yu, M. Qiao, Y. Xu, K. Setty, J. Zhang, Y. Zhu, Q. Lin, Dioxin-like compounds in agricultural soils near e-waste recycling sites from Taizhou area, China: chemical and bioanalytical characterization, Environ. Int. 35 (2009) 50–55.
[42] N. Silver, S. Best, J. Jiang, S.L. Thein, Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR, BMC Mol. Biol. 7 (2006) 33.
[43] T. Suzuki, P.J. Higgins, D.R. Crawford, Control selection for RNA quantitation, Biotechniques 29 (2000) 332–337.
[44] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002). RESEARCH0034.
[45] D. Waggott, K. Chu, S. Yin, B.G. Wouters, F.F. Liu, P.C. Boutros, NanoStringNorm: an extensible R package for the pre-processing of NanoString mRNA and miRNA data, Bioinformatics 28 (2012) 1546–1548.
[46] N. Weisglas-Kuperus, S. Patandin, G.A. Berbers, T.C. Sas, P.G. Mulder, P.J. Sauer, H. Hooijkaas, Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children, Environ. Health Perspect. 108 (2000) 1203–1207.
[47] C.Q. Yao, S.D. Prokopec, J.D. Watson, R. Pang, C. P'ng, L.C. Chong, N.J. Harding, R. Pohjanvirta, A.B. Okey, P.C. Boutros, Inter-strain heterogeneity in rat hepatic transcriptomic responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), Toxicol. Appl. Pharmacol. 260 (2012) 135–145.
[48] R. Yin, X. Liu, C. Liu, Z. Ding, X. Zhang, F. Tian, W. Liu, J. Yu, L. Li, M. Hrabe de Angelis, T. Stoeger, Systematic selection of housekeeping genes for gene expression normalization in chicken embryo fibroblasts infected with Newcastle disease virus, Biochem. Biophys. Res. Commun. 413 (2011) 537–540.